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Tumorigenesis and Neoplastic Progression

MYCN Promotes the Expansion of Phox2B-Positive Neuronal Progenitors to Drive Neuroblastoma Development

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Amplification of the oncogene *MYCN* is a tumorigenic event in the development of a subset of neuroblastomas that commonly consist of undifferentiated or poorly differentiated neuroblasts with unfavorable clinical outcome. The cellular origin of these neuroblasts is unknown. Additionally, the cellular functions and target cells of MYCN in neuroblastoma development remain undefined. Here we examine the cell types that drive neuroblastoma development in *TH-MYCN* transgenic mice, an animal model of the human disease. Neuroblastoma development in these mice begins with hyperplastic lesions in early postnatal sympathetic ganglia. We show that both hyperplasia and primary tumors are composed predominantly of highly proliferative Phox2B$^+$ neuronal progenitors. MYCN induces the expansion of these progenitors by both promoting their proliferation and preventing their differentiation. We further identify a minor population of undifferentiated nestin$^+$ cells in both hyperplastic lesions and primary tumors that may serve as precursors of Phox2B$^+$ neuronal progenitors. These findings establish the identity of neuroblasts that characterize the tumor phenotype and suggest a cellular pathway by which MYCN can promote neuroblastoma development. ***(Am J Pathol 2009, 175:856–866; DOI: 10.2353/ajpath.2009.090019)***

Neuroblastoma is a common childhood malignant tumor of the sympathetic nervous system, arising in paravertebral sympathetic ganglia and the adrenal medulla.[1] Both tissues originate from neural crest cells, a transient, highly migratory population of multipotent stem cells.[2,3] During embryonic development, neural crest cells, mainly from the trunk region of the neural crest, migrate ventrally and aggregate adjacent to the dorsal aorta to form the primary sympathetic chain. A subpopulation of cells in the primary sympathetic chain then migrate in a dorsal direction to form the secondary (definitive) sympathetic chains composed of paravertebral sympathetic ganglia, while another subpopulation of cells migrate into the adrenal gland to give rise to the adrenal medulla.[4,5]

Neuroblastoma is a heterogeneous group of tumors, displaying histological features that range from tumors with predominantly undifferentiated or poorly differentiated neuroblasts to those largely consisting of fully differentiated neurons.[1] This observation suggests that neuroblastoma may arise as the result of deregulated sympathetic neurogenesis. The molecular mechanism for the control of sympathetic neurogenesis has been largely elucidated. In response to bone morphogenetic proteins produced and secreted by the dorsal aorta, sympathetic neural crest cells express the pro-neural genes *Mash1* and *Phox2B* and adopt a neuronal fate. Mash1 and Phox2B in turn promote further neuronal differentiation by up-regulating, either directly or indirectly, the expression of transcription factors HAND2, Phox2A, and GATA3. The five transcription factors collaborate in a complex regulatory network to specify the noradrenergic phenotype of sympathetic neurons by inducing the expression of tyrosine hydroxylase (TH) and dopamine β-hydroxylase, two essential enzymes in catecholamine biosynthesis.[5,6] A critical question is at what stage of the neurogenic process neuroblastoma may arise. It is generally thought that neuroblastoma originates from primitive sympathetic progenitor cells,[1,7] but their identity remains unknown.

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Identifying the cell types that drive neuroblastoma development may not only advance our understanding of the cellular basis of the tumorigenic process but may also suggest cellular targets for therapy.

Amplification of the oncogene *MYCN* occurs in ~22% of neuroblastoma cases and is associated with advanced stages of the disease and poor prognosis.[1] Importantly, transgenic mice carrying human *MYCN* under the control of the rat *TH* gene promoter, which is active in migrating neural crest cells, develop neuroblastomas that closely resemble the human disease,[8] demonstrating that aberrant activation of *MYCN* can be an initiating event in neuroblastoma development. However, despite the early recognition of *MYCN* as an important oncogene in the pathogenesis of neuroblastoma, the cellular mechanisms for the oncogenic activity of MYCN in the tumorigenic process remain to be defined in an *in vivo* system.

It has recently been reported that neuroblastoma development in *TH-MYCN* mice begins with multifocal hyperplasia in early postnatal sympathetic ganglia.[9] The hyperplastic lesions are characterized as clusters of small round blue cells with H&E staining and are morphologically similar to human *in situ* neuroblastoma observed in the adrenal medulla of infants younger than 3 months.[10] We reasoned that a detailed examination of stage- and lineage-specific gene expression in cells comprising hyperplasia and primary tumors in *TH-MYCN* mice might shed light on the identity of progenitor cell types that drive neuroblastoma initiation and progression, and reveal the cellular basis of MYCN action in the pathogenesis of neuroblastoma.

Materials and Methods

Mice

TH-MYCN transgenic mice[8] on the 129 × 1/SvJ genetic background were obtained from the Mouse Models of Human Cancers Consortium at the National Cancer Institute-Frederick. All experiments were conducted with hemizygous *TH-MYCN* transgenic mice and their wild-type littermates. NOD.SCID/NCr mice were purchased from the National Cancer Institute-Frederick. Animals were maintained under specific pathogen-free conditions at the animal facility of University of Toledo Health Science Campus. All animal studies were pre-approved by the Institutional Animal Care and Use Committee of University of Toledo Health Science Campus.

Histology

For examination of hyperplasia in sympathetic ganglia, at least 30 mice for each genotype were euthanized by CO2 inhalation during the first 3 weeks after birth. Sympathetic ganglia, mainly superior cervical ganglia (SCG) and celiac ganglia, were collected, fixed in 10% neutral buffered formalin, embedded in paraffin blocks, sectioned at 4 μm, and stained with H&E. Each ganglion was examined for the presence of hyperplastic lesions, defined as clusters of more than 30 small blue round cells.[9] Wild-type (n = 23) and *TH-MYCN* (n = 26) mice were monitored for tumor development. Mice in a moribund state were euthanized by CO2 inhalation and examined for the presence of tumors. Each tumor was removed for histological examination by H&E staining.

Immunohistochemistry and Immunofluorescence

Paraffin embedded mouse sympathetic ganglia or primary tumors were sectioned at 4 μm, deparaffinized, and rehydrated. For antigen retrieval, sections were treated for 20 minutes at 95°C in 10 mmol/L citrate buffer (pH 6.0) in a laboratory microwave oven and subsequently washed in PBS. For immunohistochemistry, after quenching of endogenous peroxidase activity and blocking with normal goat serum, sections were incubated sequentially with primary antibodies, biotinylated goat anti-mouse or anti-rabbit IgG, and the ABC reagent (Vector Laboratories). The immunostaining was visualized with 3,3′-diaminobenzidine (Sigma). Sections were then counterstained with hematoxylin before being examined using a light microscope. For immunofluorescence, tissue sections were stained overnight with primary antibodies at 4°C, incubated with secondary antibodies for 2 hours at room temperature, counterstained with 4,6-diamidino-2-phenylindole (DAPI) and mounted with a fluorescence mounting medium (Dako) before examination using a Nikon ECLIPSE E800 fluorescence microscope (Nikon Instruments) or an Olympus IX81 Spinning Disk Confocal microscope (Olympus America). Staining with mouse monoclonal antibodies was performed using the Mouse-on-Mouse kit to reduce background staining according to the manufacturer's instructions (Vector laboratories). The following primary antibodies were used in immunohistochemistry and immunofluorescence: mouse anti-MYCN (1:100, clone AB-1, Oncogene Research), mouse anti-Ki-67 (1:100, clone B56, BD Pharmingen), rabbit anti-phosphorylated histone H3 (pHH3) (1:500, Upstate), rabbit anti-Phox2B (1:600, kindly provided by Jean-FranÇois Brunet),[11] mouse anti-Mash1 (1:100, clone 24B72D11.1, BD Pharmingen),[12] mouse anti-TH (1:5000, Sigma), rabbit anti-TH (1:1000, Chemicon), rabbit anti–brain lipid-binding protein (BLBP, 1:2000, Chemicon), chicken anti-nestin (1:1000, Novus Biologicals), and rabbit anti-S100 (1:200, Dako). All secondary fluorescence antibodies were from Molecular Probes and used at 1:400 dilutions: goat anti-mouse (fluorescein isothiocyanate or Texas-Red), goat anti-rabbit (Alexa Fluor 594 or fluorescein isothiocyanate), and goat anti-chicken (Alexa Fluor 488).

Immunoblotting

Human neuroblastoma cell lines BE(2)-C, SK-N-DZ, and SK-N-AS were directly suspended in sodium dodecyl sulfate sample buffer, and 50 μg of proteins were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel, transferred to a nitrocellulose membrane, probed with mouse anti-MYCN (NCM II 100, 1:200, kindly provided by Naohiko Ikegaki)[13] or mouse anti-α-tubulin (B-5–1-2, 1:2000, Sigma), and visualized by chemiluminescence (SuperSignal West Femto Chemiluminescent kit, Pierce).

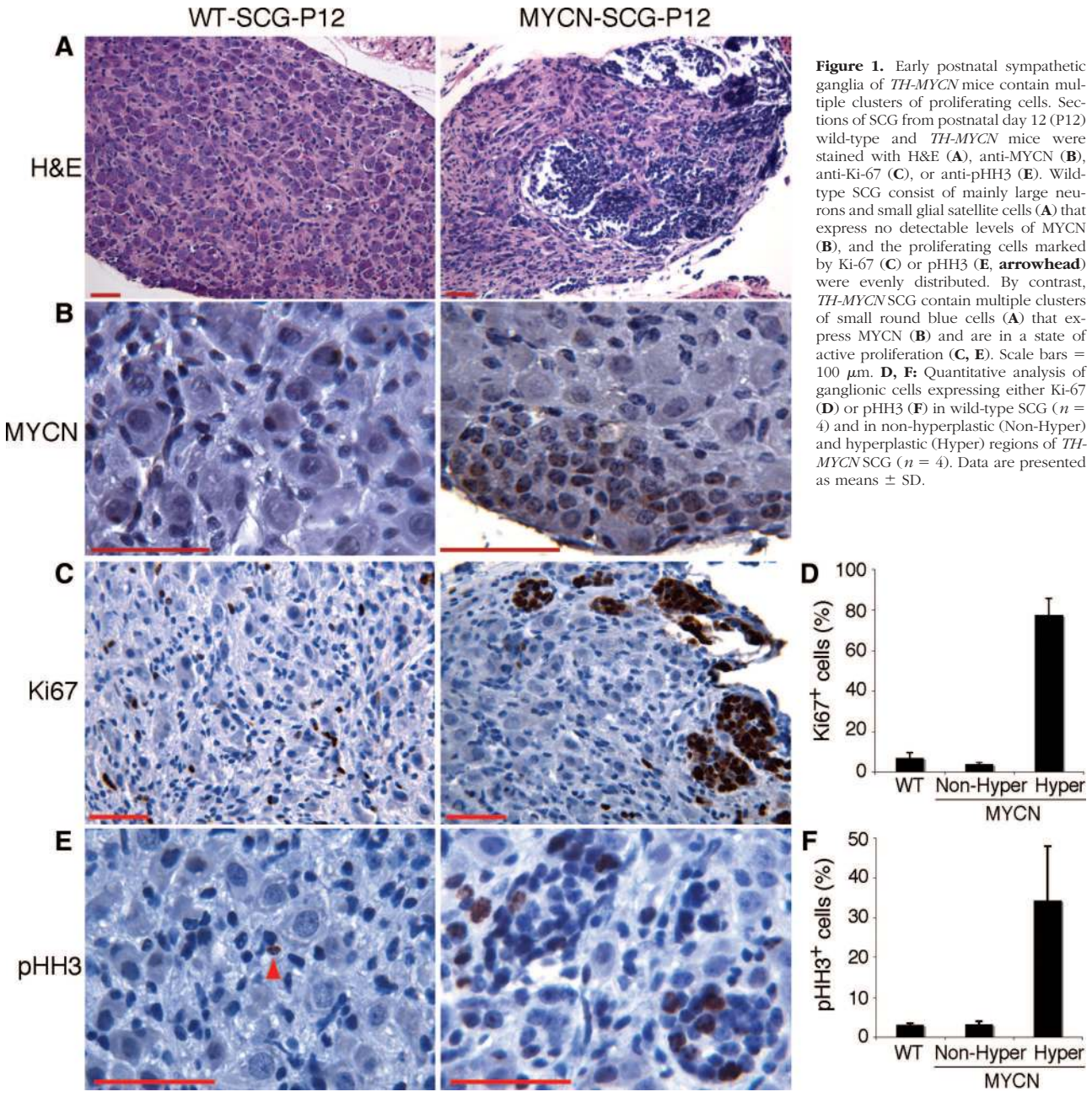

Figure 1. Early postnatal sympathetic ganglia of *TH-MYCN* mice contain multiple clusters of proliferating cells. Sections of SCG from postnatal day 12 (P12) wild-type and *TH-MYCN* mice were stained with H&E (**A**), anti-MYCN (**B**), anti-Ki-67 (**C**), or anti-pHH3 (**E**). Wild-type SCG consist of mainly large neurons and small glial satellite cells (**A**) that express no detectable levels of MYCN (**B**), and the proliferating cells marked by Ki-67 (**C**) or pHH3 (**E**, **arrowhead**) were evenly distributed. By contrast, *TH-MYCN* SCG contain multiple clusters of small round blue cells (**A**) that express MYCN (**B**) and are in a state of active proliferation (**C, E**). Scale bars = 100 μm. **D, F:** Quantitative analysis of ganglionic cells expressing either Ki-67 (**D**) or pHH3 (**F**) in wild-type SCG (n = 4) and in non-hyperplastic (Non-Hyper) and hyperplastic (Hyper) regions of *TH-MYCN* SCG (n = 4). Data are presented as means ± SD.

Cell Culture and Xenograft Assay

Human neuroblastoma cell lines SK-N-AS and SK-N-DZ were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and BE(2)-C in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 supplemented with 10% fetal bovine serum and nonessential amino acids. Six NOD. SCID/NCr mice (6 to 8 weeks old) were used for each cell line, and for each mouse, both flanks were injected subcutaneously with 5×10^6 cells in 200 μl of Dulbecco's modified Eagle's medium. Tumor growth was estimated by caliper measurements and tumor volume was calculated with the formula $4/3\pi r^3$, where r is the radius of the tumor.[14] Two weeks after injection, tumors formed at the injection sites were removed, weighed, and paraffin-embedded. Tumor sections were stained with H&E for histological examination or antibodies for marker expression analyses.

Quantification and Statistics

For tissue sections, approximately 1000 cells (DAPI-positive) were counted from at least four randomly selected ×400 fields, and the percentages of nestin$^+$, Phox2B$^+$, Ki-67$^+$ and/or pHH3$^+$ cells were determined. Data were presented as mean ± SD. Statistical analysis was performed using two-tailed Student's t-test. P value <0.05 is considered statistically significant.

Results

Early Postnatal Sympathetic Ganglia of TH-MYCN Mice Contain Multiple Clusters of Proliferating Cells

To visualize the early stage of neuroblastoma development, we isolated and examined SCG and celiac ganglia from *TH-MYCN* mice of 1 to 3 weeks of age. As controls, we also examined sympathetic ganglia isolated from age-matched wild-type littermates. Wild-type sympathetic ganglia consist of two major cell types, large sympathetic neurons and small glial cells (Figure 1A). Approximately 7% of postnatal sympathetic ganglia in wild-type mice contained small clusters of round blue cells (data not shown). Consistent with the previous report,[9] we observed the presence of multiple clusters of small round blue cells in approximately 36% of early postnatal sympathetic ganglia of *TH-MYCN* mice (Figure 1A). Immunohistochemical staining revealed that only hyperplastic cells in *TH-MYCN* sympathetic ganglia expressed detectable levels of MYCN (Figure 1B).

To understand the cellular basis for the formation of hyperplastic lesions in sympathetic ganglia of *TH-MYCN* mice, we examined the proliferation status of hyperplastic cells, with age-matched wild-type ganglionic cells as control. We first performed immunohistochemical staining for the proliferation marker Ki-67, a nuclear protein specifically expressed in cells undergoing active proliferation.[15] Wild-type sympathetic ganglia contained a significant number of Ki-67$^+$ cells (6.9% of total cells) that were evenly distributed, and no specialized proliferation zones or areas were observed (Figure 1, C and D). Also, approximately 4% of cells in the non-hyperplastic region of *TH-MYCN* sympathetic ganglia expressed Ki-67 (Figure 1, C and D). Strikingly, an average of 77.4% of the hyperplastic cells in *TH-MYCN* sympathetic ganglia stained positively for Ki-67 (Figure 1, C and D). We next stained the tissue sections with an antibody against pHH3, a marker for mitotic cells.[16] Approximately 3.1% cells in the wild-type sympathetic ganglia were in the mitotic phase of the cell cycle, and a similar number of pHH3$^+$ cells were found in the non-hyperplastic region of *TH-MYCN* sympathetic ganglia (Figure 1, E and F). In contrast, approximately 34.3% of the hyperplastic cells stained positively for pHH3 (Figure 1, E and F). Together, these results indicate that MYCN expression promotes the proliferation of a subpopulation of cells in sympathetic ganglia, leading to the formation of hyperplastic lesions.

Hyperplastic Lesions Are Composed Predominantly of Phox2B$^+$ Neuronal Progenitors

Given their highly proliferative state and immature morphology, it appears to be likely that the hyperplastic cells are a population of progenitor cells. Although they have been referred to as "neuroblasts",[9] their identity has not been established. We first wanted to confirm that they are not of glial lineage, in light of our recent finding that the majority of Ki-67$^+$ cells in early postnatal sympathetic ganglia of wild-type mice express BLBP[17] (see also Figure 2A), a marker for glial progenitor cells in the peripheral nervous system including the sympathetic nervous system.[17,18] Immunofluorescent staining revealed that all Ki-67$^+$ hyperplastic cells in *TH-MYCN* sympathetic ganglia stained negatively for BLBP (Figure 2A), indicating that they are not glial progenitor cells.

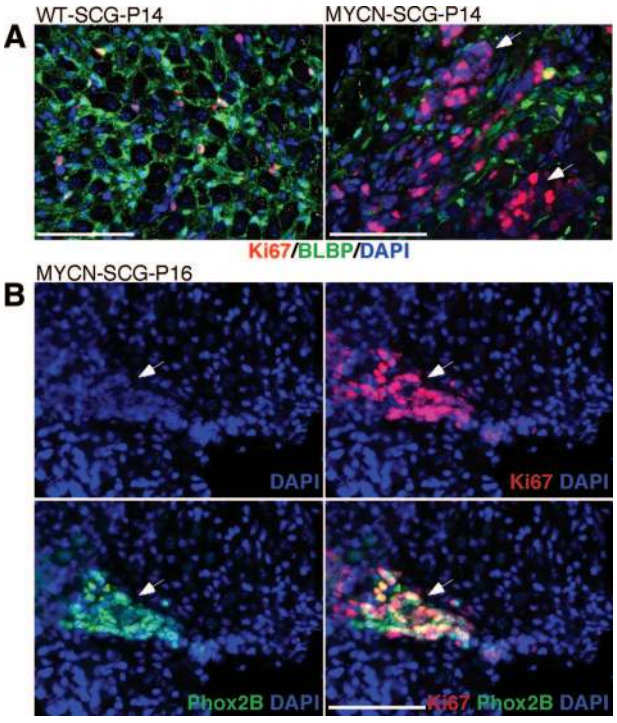

Figure 2. Hyperplastic lesions are composed predominantly of proliferating Phox2B$^+$ cells. **A:** Sections of SCG from P14 wild-type and *TH-MYCN* mice were stained with anti-Ki-67 (red) and anti-BLBP (green), an early marker for glial cells. Most of the Ki-67$^+$ cells in wild-type SCG also express BLBP, whereas Ki-67$^+$ cells in hyperplastic lesions (**arrows**) are negative for BLBP. **B:** Immunofluorescent staining of a representative SCG section from a P16 *TH-MYCN* mouse shows a hyperplastic lesion (**arrow**) consisting predominantly of cells expressing both Ki-67 (red) and Phox2B (green). Nuclei were stained with DAPI (blue). Scale bars = 100 μm.

We next stained the hyperplastic cells for markers of neuronal progenitors. Mash1 and Phox2B are among the first markers expressed in early sympathetic neuronal progenitors.[5,6] We failed to detect Mash1 expression in hyperplastic cells by immunofluorescent staining (data not shown), although the same antibody could stain a small population of cells in mouse embryonic sympathetic ganglia (data now shown). By contrast, most of the hyperplastic cells expressed Phox2B, as well as Ki-67 (Figure 2B), indicating that they are Phox2B$^+$ neuronal progenitors in an actively proliferating state. We conclude from these data that MYCN specifically promotes the proliferation of Phox2B$^+$ neuronal progenitors to initiate neuroblastoma development.

Phox2B$^+$ Progenitors in Hyperplasia Are Arrested in Neuronal Differentiation

In wild-type mice early postnatal sympathetic ganglia contained a large number of Phox2B$^+$ cells and a vast

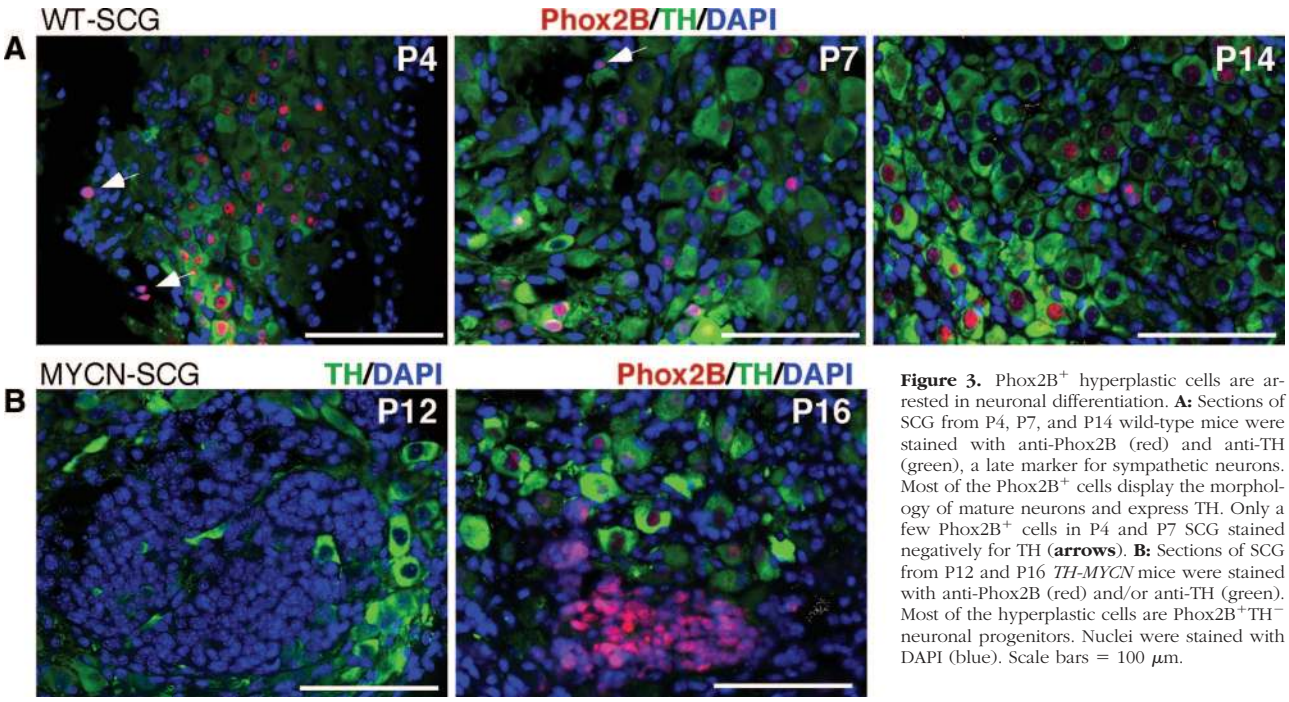

Figure 3. Phox2B$^+$ hyperplastic cells are arrested in neuronal differentiation. **A:** Sections of SCG from P4, P7, and P14 wild-type mice were stained with anti-Phox2B (red) and anti-TH (green), a late marker for sympathetic neurons. Most of the Phox2B$^+$ cells display the morphology of mature neurons and express TH. Only a few Phox2B$^+$ cells in P4 and P7 SCG stained negatively for TH (**arrows**). **B:** Sections of SCG from P12 and P16 *TH-MYCN* mice were stained with anti-Phox2B (red) and/or anti-TH (green). Most of the hyperplastic cells are Phox2B$^+$TH$^-$ neuronal progenitors. Nuclei were stained with DAPI (blue). Scale bars = 100 μm.

majority of them expressed TH, a late marker for sympathetic noradrenergic neurons, and displayed the morphology of mature sympathetic neurons (Figure 3A). We did observe a few Phox2B$^+$ cells that expressed no detectable levels of TH in postnatal day 4 (P4) and P7 sympathetic ganglia (Figure 3A, P4 and P7, arrows). However, no such cells were found in sympathetic ganglia from P14 (Figure 3A) and older mice (data not shown). Moreover, while approximately 60% of TH$^+$ sympathetic neurons expressed Phox2B at P4, the number decreased gradually with time, and by P28, only 12% of TH$^+$ sympathetic neurons showed detectable levels of Phox2B expression (data not shown). Thus, Phox2B is down-regulated during final neuronal differentiation.

By contrast, Phox2B$^+$ hyperplastic cells in MYCN sympathetic ganglia as late as P16 showed no sign of final neuronal differentiation. They displayed the morphology of undifferentiated, small round cells and expressed no detectable levels of TH (Figure 3B). These findings, coupled to the observation of their highly proliferating state, indicate that Phox2B$^+$ hyperplastic cells are arrested at the progenitor stage. Thus, MYCN not only promotes the proliferation of Phox2B$^+$ neuronal progenitors but also blocks their differentiation into TH-expressing mature neurons, resulting in marked expansion of the progenitor population in sympathetic ganglia and consequently the formation of hyperplastic lesions.

Phox2B$^+$ Progenitor Cells Are the Major Cellular Component of Primary Neuroblastoma Tumors

The data presented above suggest that MYCN-induced expansion of Phox2B$^+$ neuronal progenitors is a critical event in neuroblastoma initiation in *TH-MYCN* mice. We then asked whether expansion of the same progenitor population also drives neuroblastoma growth and progression. Histological examination revealed that neuroblastoma tumors from *TH-MYCN* mice were largely composed of small round blue cells that were morphologically similar to the hyperplastic cells observed in early postnatal sympathetic ganglia of *TH-MYCN* mice and formed apparent nests or lobules surrounded by thin fibrovascular septa (Figure 4A, H&E), characteristics of human neuroblastomas with *MYCN* amplification.[19] A majority of tumor cells stained positively for Ki-67, indicating that they were in an actively proliferating state (Figure 4A, Ki-67). Importantly, most of the tumor cells expressed Phox2B, and most of the Phox2B$^+$ tumor cells expressed no detectable levels of TH (Figure 4A, Phox2B/TH/DAPI), indicative of their undifferentiated state. Co-immunostaining of tumor sections for Ki-67 and Phox2B (Figure 4B) and quantitative analysis revealed that 70.1% of Phox2B-expressing tumor cells stained positively for Ki-67, whereas only 5.9% of Phox2B− cells expressed Ki-67. Together, these data identify Phox2B$^+$ neuronal progenitors as a major cellular component of neuroblastoma tumors developed in *TH-MYCN* mice. The data also suggest that the proliferation of undifferentiated Phox2B$^+$ progenitors is a major cellular mechanism for driving neuroblastoma growth.

Phox2B$^+$ Neuronal Progenitors Drive the Growth of Xenograft Tumors Derived from Human Neuroblastoma Cell Lines

We next addressed the question of whether our findings in *TH-MYCN* mice are relevant to the pathogenesis of human neuroblastoma. Recently it has been reported that all of the primary human neuroblastoma tumors examined

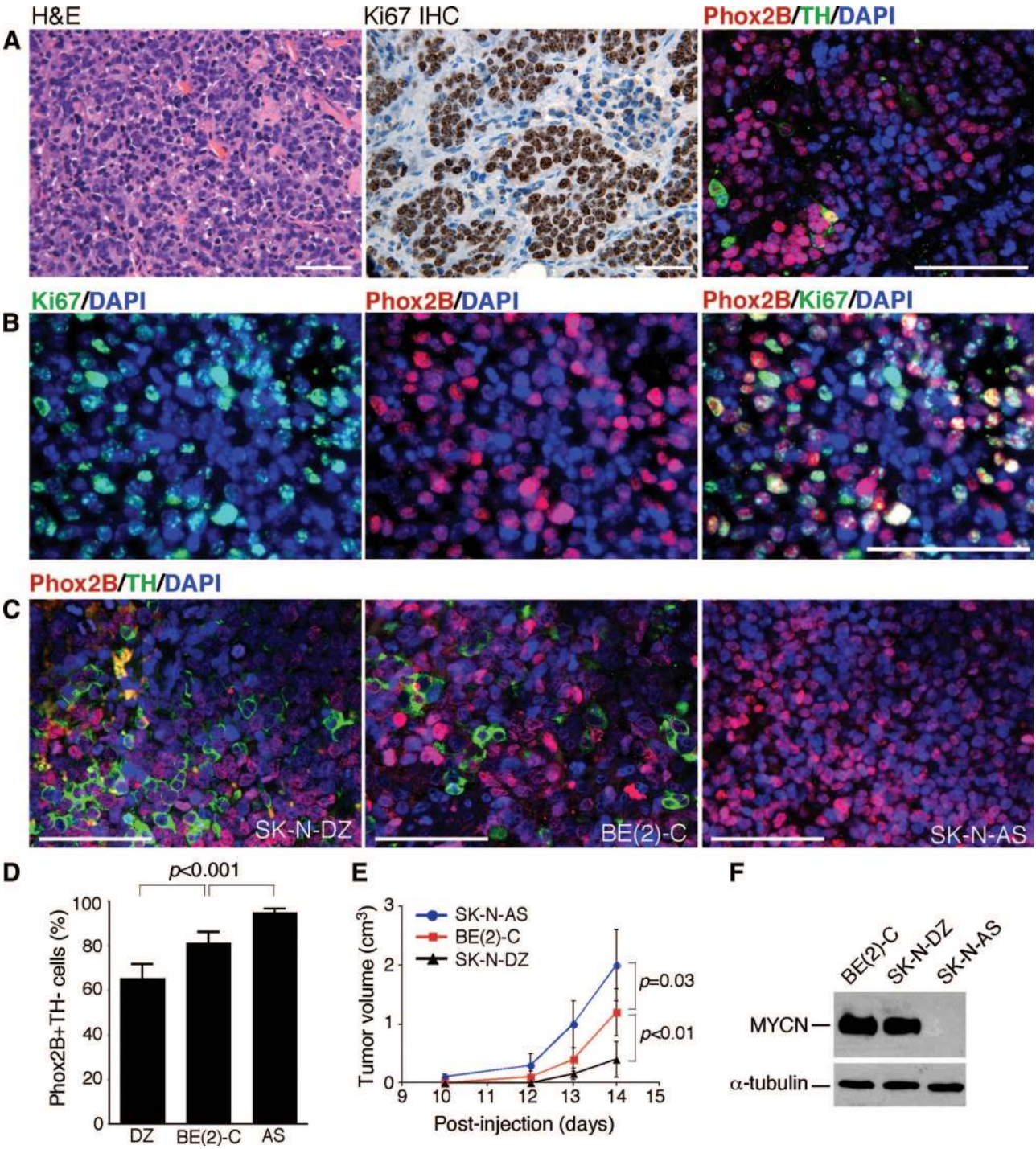

Figure 4. Phox2B$^+$ progenitor cells are the major cellular component of mouse primary neuroblastoma tumors and xenograft tumors derived from human neuroblastoma cell lines. **A:** Sections of neuroblastoma tumors from *TH-MYCN* mice were stained with H&E, anti-Ki-67, or anti-Phox2B (red) and anti-TH (green). Nuclei were stained with DAPI (blue). The small round blue tumor cells are organized in nests surrounded by thin fibrovascular septa (H&E), and most of them express Ki-67 and Phox2B, but are negative for TH. **B:** Immunofluorescent staining of a representative mouse neuroblastoma section for Ki-67 (green) and Phox2B (red). Nuclei were stained with DAPI (blue). **C:** Sections of xenograft tumors derived from human neuroblastoma cell lines were stained with anti-Phox2B (red) and anti-TH (green). Nuclei were stained with DAPI (blue). Most of the tumor cells express Phox2B. SK-N-DZ and BE(2)-C xenografts also contain significant numbers of TH$^+$ cells. The cells stained yellow in SK-N-DZ xenograft section are probably necrotic cells. Scale bars = 100 μm (**A-C**). **D, E:** Quantification of Phox2B$^+$TH$^-$ progenitor cells (**D**) in xenograft tumors of different human neuroblastoma cell lines reveals a positive correlation with the tumor growth rates (**E**). Data are presented as means ± SD and analyzed with two-tailed Student's *t*-test with *P* values indicated. **F:** Immunoblot analysis of MYCN protein levels in human neuroblastoma cell lines. α-tubulin levels are shown as loading control.

express high levels of Phox2B mRNA, determined by microarray and quantitative reverse transcription-PCR analyses.[20] Consistent with the finding, immunofluorescent staining showed that xenograft tumors derived from human neuroblastoma cell lines consisted largely of Phox2B$^+$ cells (Figure 4C). Quantitative analysis indicated that Phox2B$^+$ neuronal progenitors, defined by their lack of TH expression, comprised 65.6%, 81.5%,

and 94.8% of cells in xenograft tumors derived from SK-N-DZ, BE(2)-C, and SK-N-AS cell lines, respectively (Figure 4D). Importantly, xenograft tumors with higher numbers of Phox2B$^+$ progenitor cells grew significantly faster than those with lower numbers of Phox2B$^+$ progenitor cells (Figure 4E), suggesting a critical role of Phox2B$^+$ progenitor cells in driving the tumor growth. These findings, coupled to the report that primary human neuroblastoma tumors express abundant Phox2B mRNA,[20] suggest that expansion of Phox2B$^+$ neuronal progenitors is also critical for the pathogenesis of human neuroblastomas.

It should be pointed out that SK-N-DZ and BE(2)-C are *MYCN*-amplified cell lines and express high levels of MYCN, whereas SK-N-AS cells carry single-copy *MYCN* and express little or no detectable level of MYCN (Figure 4F). This observation suggests the existence of MYCN-independent mechanisms for promoting the expansion of Phox2B$^+$ progenitor cells during human neuroblastoma development, which is consistent with the notion that expansion of Phox2B$^+$ progenitor cells is a common event in the pathogenesis of human neuroblastoma, regardless of the mechanisms involved.

Hyperplasia and Primary Neuroblastoma Tumors Contain a Minor Population of Nestin$^+$ Cells with Progenitor Features

During our investigation, we noted that hyperplastic lesions also contained a minor population of cells that stained negatively for Phox2B or other lineage markers such as BLBP, S100, and TH, suggesting that they may represent a distinct progenitor population. To uncover their identity, we performed immunofluorescent staining of hyperplastic cells for the expression of Phox2B and nestin, a filament protein expressed in a variety of tissue stem cells and progenitor cells including neural crest cells[21] (Cui and Ding, unpublished data). Nestin is also expressed in sympathetic glial and neuronal progenitors.[17] Our immunofluorescent staining revealed the presence of three distinct cell populations in hyperplasia: nestin$^+$, Phox2B$^+$, and nestin$^+$Phox2B$^+$ cells (Figure 5A). Given their physical location and the fact that nestin is expressed in neural crest cells that give rise to sympathetic glia and neurons, it appears to be likely that the three cell populations represent distinct stages of sympathetic neurogenesis, with nestin$^+$ cells being precursors of the nestin$^+$Phox2B$^+$ cells that in turn differentiate into Phox2B$^+$ neuronal progenitors.

Quantitative analysis revealed a fourfold increase in the number of nestin$^+$ cells in MYCN sympathetic ganglia with hyperplastic lesions in comparison with age-matched, wild-type, sympathetic ganglia (Figure 5, B and C). It should be pointed out that the majority of nestin$^+$ cells in early postnatal sympathetic ganglia of wild-type mice are glial progenitors that express BLBP and differentiate into satellite cells.[17] By contrast, nestin$^+$ cells in the hyperplastic lesions expressed either no lineage markers or Phox2B, a neuronal marker. Taken together, these data suggest that MYCN expression not only results in an increase in the number of nestin$^+$ sympathetic progenitors, but also biases them toward a neuronal fate. A combination of these effects probably also contributes to the expansion of Phox2B$^+$ neuronal progenitors and the formation of neuroblast hyperplasia observed in sympathetic ganglia of *TH-MYCN* mice.

Similar to what observed in hyperplastic lesions, nestin$^+$ cells were also present in primary neuroblastoma tumors from *TH-MYCN* mice and comprised approximately 4.6% of cells in all tumor samples examined (Figure 5D). Immunofluorescent staining for lineage markers revealed that nestin$^+$ tumor cells expressed no TH or BLBP (Figure 5D), but some of them stained positively for Phox2B (Figure 5E). These findings are consistent with the suggestion that the nestin single-positive tumor cells may represent a population of malignant sympathetic neural crest cells or early progenitors that give rise to the Phox2B$^+$ tumor cells comprising the bulk of neuroblastoma tumors.

Discussion

Using *TH-MYCN* transgenic mice as a model system, we show that MYCN induces marked expansion of Phox2B$^+$ neuronal progenitors in sympathetic ganglia to drive neuroblastoma initiation and progression. The expansion is caused by enhanced proliferation and arrested differentiation of Phox2B$^+$ progenitors, with possible contribution from an increase in the pool of nestin$^+$ cells that may serve as precursors of Phox2B$^+$ neuronal progenitors. We further present evidence suggesting that expansion of Phox2B$^+$ neuronal progenitors may also drive the growth of human neuroblastomas. These findings shed light on the cellular basis of neuroblastoma development and define the cellular functions of MYCN in the tumorigenic process, with important clinical implications.

The Cellular Functions of MYCN in the Pathogenesis of Neuroblastoma

The oncogene *MYCN* was originally identified in neuroblastoma cells.[22,23] Like other members of the MYC family, MYCN regulates important cellular processes including proliferation, growth, metabolism, apoptosis, and differentiation.[24,25] However, which of these cellular functions of MYCN is critical for neuroblastoma development has not been rigorously defined, especially under *in vivo* conditions. We address this question using *TH-MYCN* mice as an experimental system. Neuroblastoma development in *TH-MYCN* mice begins with hyperplastic lesions in sympathetic ganglia during the first few weeks after birth. Our investigation reveals that the hyperplastic lesions are composed predominantly of Phox2B$^+$ neuronal progenitors with elevated levels of MYCN protein. A majority of the Phox2B$^+$ progenitor cells are in a state of active proliferation, as evidenced by their expression of Ki-67. Thus, sustained MYCN expression specifically promotes the proliferation of Phox2B$^+$ neuronal progenitors, contributing to the formation of hyperplasia.

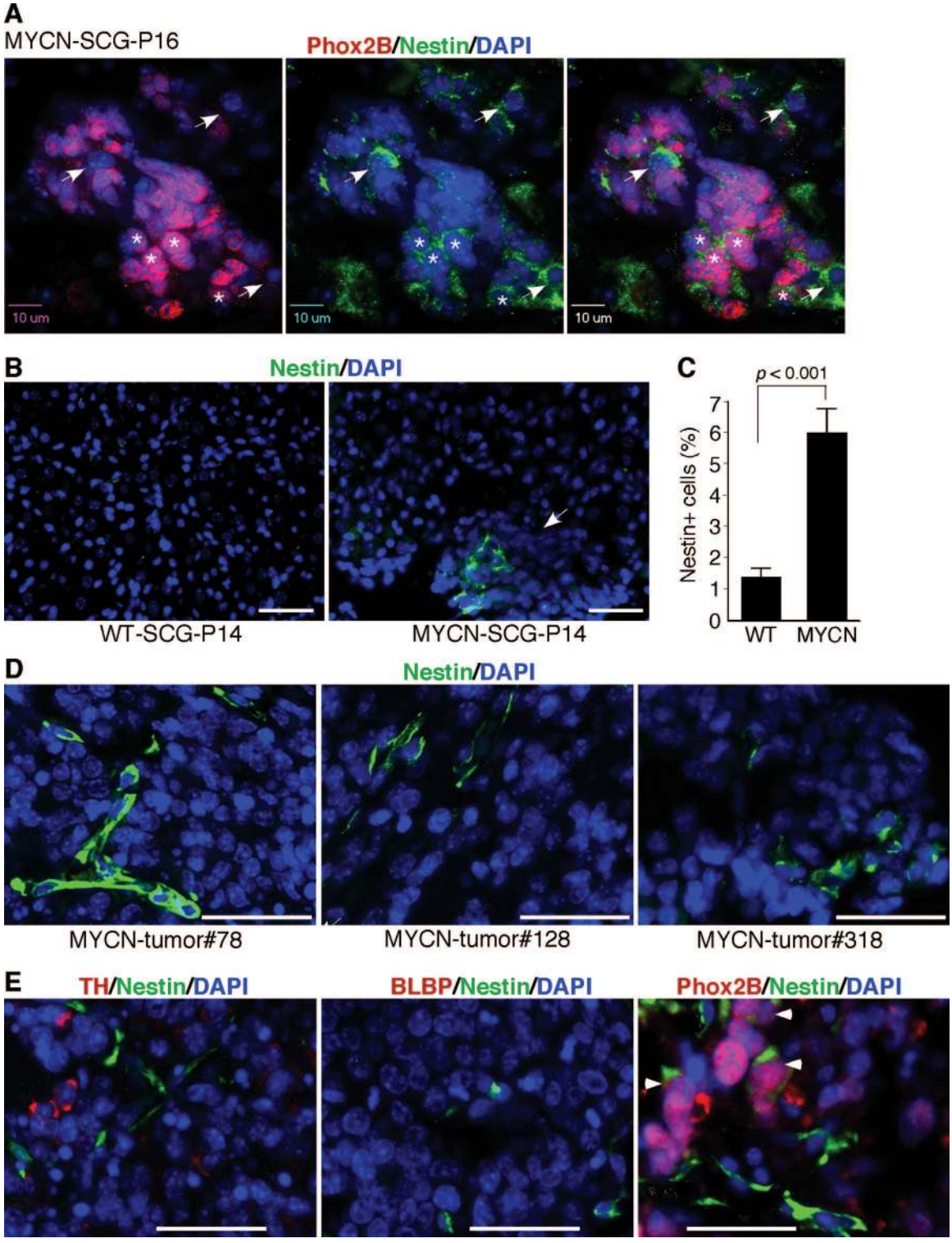

Figure 5. Hyperplasia and primary neuroblastoma tumors contain a minor population of nestin$^+$ progenitor cells. **A:** Sections of hyperplastic lesions were stained with anti-Phox2B (red), anti-nestin (green), and DAPI (blue), and examined with a confocal microscope. **Arrows** and **asterisks** indicate nestin$^+$ and nestin$^+$Phox2B$^+$ cells, respectively. **B:** Sections of wild-type and MYCN sympathetic ganglia at P14 were stained with anti-nestin (green) and DAPI (blue). The **arrow** indicates a hyperplastic lesion in the MYCN SCG. **C:** Quantitative analysis showing a fourfold increase in the number of nestin$^+$ cells in *TH-MYCN* SCG with hyperplastic lesions compared with age-matched wild-type SCG (P14 to P16). Data are presented as means ± SD and analyzed by two-tailed Student's *t*-test with the *P* value indicated. **D, E:** Sections of representative primary neuroblastoma tumors from *TH-MYCN* mice were stained with anti-nestin (green) alone (**D**) or with anti-TH (red), anti-BLBP (red) or anti-Phox2B (red) (**E**). Nuclei were stained with DAPI (blue). Nestin$^+$ tumor cells are negative for TH and BLBP, and some of them express Phox2B (**arrowheads**). Scale bars: 10 μm (**A**); 50 μm (**B, D,** and **E**).

The hyperplastic lesions also contain a population of nestin$^+$ cells. Some of them have committed to a neuronal fate as evidenced by their expression of Phox2B, while others express no detectable levels of lineage markers and may represent a population of primitive sympathetic progenitors or neural crest cells. This is in striking contrast to the nestin$^+$ cells found in sympathetic ganglia of wild-type mice during the first 2 weeks after birth. Most of these nestin$^+$ cells express BLBP and differentiate into sympathetic satellite cells.[17] These observations suggest that MYCN may bias nestin$^+$ sympathetic progenitors toward a neuronal fate versus a glial fate, thereby contributing to the expansion of Phox2B$^+$ neuronal progenitors. This model is consistent with data from several studies. *MYCN*$^{-/-}$ mice have a significant reduction in the number of neurons, but not glial cells, in sympathetic ganglia and other nervous tissues originating from neural crest cells.[26–28] Transient overexpression of MYCN in avian neural crest cells increases the generation of neurons.[29] Moreover, conditional knockout of the *MYCN* gene in the central nervous system demonstrates an essential role of MYCN in driving expansion of neural progenitor populations during embryonic neurogenesis,[30] indicating a more general role for MYCN in regulation of neuronal fate decisions. This pro-neuronal function of MYCN may be critical for its oncogenic activity in the pathogenesis of tumors of neuronal origin including neuroblastomas,[22,23] medulloblastomas,[31] and retinoblastomas,[32] as noted previously.[30]

It has been proposed that arrested differentiation of neuroblasts is an early event in the pathogenesis of neuroblastoma, with possible involvement of MYCN, based on *in vitro* studies of human neuroblastoma cell lines.[33,34] Our study provides the first line of direct *in vivo* evidence in support of this model and defines the specific stage at which the differentiation process is blocked. We show that during early postnatal sympathetic development in wild-type mice, Phox2B$^+$ neuronal progenitors gradually differentiate into morphologically mature neurons with high-level expression of TH. The differentiation process is essentially completed by P14. By contrast, Phox2B$^+$ cells in hyperplastic lesions are maintained in a progenitor state. Also, most of Phox2B$^+$ cells in neuroblastoma tumors from *TH-MYCN* mice express no detectable levels of TH. Thus, MYCN blocks the differentiation process at the stage defined by Phox2B expression, leading to further expansion of the progenitor pool.

It remains a puzzle how MYCN expression driven by the rat *TH* promoter leads to the expansion of a population of neuronal progenitors with no significant levels of TH expression. Many factors, including the local chromatin structure of the transgene integration site may influence the activity of the promoter, rendering it active in sympathetic progenitor cells. Once transformed by MYCN, these cells are arrested in an undifferentiated state, thereby losing the ability to express differentiation markers such as TH.

On the basis of the above discussion, we suggest a model for the cellular functions of MYCN in the pathogenesis of neuroblastoma (Figure 6). Oncogenic activation (amplification or overexpression) of MYCN promotes the commitment of nestin$^+$ progenitor cells to the neuronal lineage marked by Phox2B expression, leading to an increase in the pool of Phox2B$^+$ neuronal progenitors. The progenitor cell pool is then further expanded through enhanced proliferation and arrested differentiation mediated by MYCN, which initiates and drives neuroblastoma development. This model also implies that Phox2B$^+$ neuronal progenitors may provide a critical cellular context for the oncogenic activity of MYCN in the pathogenesis of neuroblastoma.

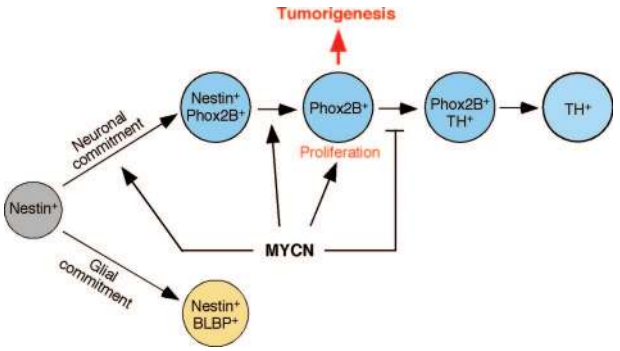

Figure 6. A simplified schematic diagram for the cellular pathway in MYCN-induced neuroblastoma development. See text for discussion.

The Cellular Basis of Neuroblastoma Development

A central tenet of the cancer stem cell model is that cancer development can be viewed as abnormal organogenesis initiated by malignant transformation of a tissue stem cell or progenitor cell.[35] Normal sympathetic neurogenesis is initiated with the commitment of sympathetic neural crest cells to a neuronal lineage as the result of bone morphogenetic protein-induced expression of Mash1 and Phox2B. The two transcription factors then activate the expression of Phox2A, HAND2, and GATA3, which in turn drive neuronal progenitors to undergo final differentiation into mature noradrenergic neurons marked by high-level expression of TH and other enzymes in catecholamine biosynthesis.[5,6]

Neuroblastoma development in *TH-MYCN* mice appears to recapitulate the neurogenic process, but with a block at the progenitor stage defined by Phox2B expression. Our study reveals that hyperplastic lesions in early postnatal sympathetic ganglia of *TH-MYCN* mice contain three distinct cell populations: nestin$^+$, Phox2B$^+$, and nestin$^+$Phox2B$^+$ cells. Nestin is an intermediate filament originally identified in neural stem cells,[36] and is expressed in neural crest cells isolated from rodent embryonic trunk neural tubes[21] (Cui and Ding, unpublished data) and in neural crest cells derived from human embryonic stem cells.[37] More recently, we have shown that nestin expression is associated with the progenitor state of lineage-restricted sympathetic progenitor cells.[17] On the basis of these observations, we suggest that the nestin$^+$ cells in hyperplastic lesions are a population of sympathetic neural crest cells or primitive progenitors that give rise to first nestin$^+$Phox2B$^+$ and then Phox2B$^+$

neuronal progenitors. Along this line of reasoning and in light of the observed expansion of nestin$^+$ cells in hyperplastic lesions, we further suggest that nestin$^+$ cells in sympathetic ganglia are probably the cells of origin for neuroblastoma in *TH-MYCN* mice.

However, given that nestin$^+$ cells only constitute 6% and 4.6% of the cells in hyperplastic lesions and primary neuroblastoma tumors, respectively, it seems clear that the tumor growth is driven largely by Phox2B$^+$ progenitor cells, the predominant cell type in both hyperplastic lesions and primary tumors. A majority of the Phox2B$^+$ progenitor cells express Ki-67, indicating that they are in a state of active proliferation. Moreover, the numbers of Phox2B$^+$ progenitor cells within xenograft tumors correlate positively with the tumor growth rates. Collectively, our data suggest that Phox2B$^+$ neuronal progenitors are the major cellular target of MYCN in driving neuroblastoma development from hyperplasia to tumors (Figure 6). These findings further suggest that the neuronal lineage commitment defined by Phox2B expression is a critical event in MYCN-induced neuroblastoma development.

Our data may also have significance for human neuroblastoma. It has been shown recently that all primary human neuroblastoma tumors examined express high levels of Phox2B mRNA,[20] and we found that the majority of tumor cells in xenografts derived from human neuroblastoma cell lines express Phox2B, regardless of the status of *MYCN* amplification. Together, these data suggest that expansion of Phox2B$^+$ neuronal progenitors is a common cellular event in the pathogenesis of neuroblastoma.

Human neuroblastomas of advanced stages are characterized histologically as tumors composed predominantly of undifferentiated or poorly differentiated neuroblasts with a high mitotic rate.[38,39] These tumors commonly carry *MYCN* amplification.[19] Our study establishes the identity of the neuroblasts as Phox2B$^+$ neuronal progenitors. In addition, we show that MYCN specifically promotes the expansion of Phox2B$^+$ neuronal progenitors. These findings provide a mechanistic understanding of the phenotype of human neuroblastomas with *MYCN* amplification and suggest that targeting Phox2B$^+$ progenitor cells could be a valuable strategy for clinical treatment of neuroblastomas of advanced stages. Phox2B might also be useful as a biomarker for neuroblastoma staging, prognosis, and treatment selection.

The finding that Phox2B$^+$ neuronal progenitors are the major cellular component of both hyperplastic lesions and neuroblastoma tumors suggests a possible role for Phox2B in the tumorigenic process. Phox2B is a homeodomain-containing transcription factor essential for the generation of noradrenergic neurons of the sympathetic nervous system, and mice with homozygous inactivation of *Phox2B* fail to develop sympathetic ganglia.[40] Consistent with a role of Phox2B in neuroblastoma development, several studies have reported heterozygote germline mutations of *Phox2B* in hereditary neuroblastoma.[41–43] Moreover, we recently observed that retinoic acid-induced differentiation of human neuroblastoma cell lines was accompanied by marked down-regulation of Phox2B (Alam and Ding, unpublished data), suggesting that Phox2B might be critical for the maintenance of neuroblasts that characterize neuroblastoma tumors. However, overexpression of Phox2B in human neuroblastoma cell lines has been shown to suppress proliferation and promotes neuronal differentiation.[20] We speculate that *Phox2B* may function as a lineage-dependent or lineage-survival oncogene in neuroblastoma development, analogous to the role of microphthalmia-associated transcription factor in the pathogenesis of melanoma.[44] Microphthalmia-associated transcription factor is a master regulator of melanocyte differentiation and survival.[45] It promotes melanocyte differentiation by inducing growth arrest,[46,47] but can cooperate with activated BRAF to transform primary human melanocytes.[48] Thus, it is possible that Phox2B only displays its oncogenic activity in the context of deregulation of cell cycle and differentiation, such as aberrant activation of MYCN. This possibility is currently under investigation. Our initial findings suggest that Phox2B is probably not a direct target gene of MYCN (Alam and Ding, unpublished data). Nevertheless, emerging evidence suggests that the cooperation between oncogenic proteins and lineage-determining factors might be an important mechanism underlying the development of tissue-type specific tumors.[49,50]

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